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Review

A Review of the Mechanisms Involved in the Action of Phosphine as an Insecticide and Phosphine Resistance in Stored-Product Insects

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Abstract: Phosphine gas has been used world-wide for more than four decades as an ideal fumigant for disinfestation of stored grains and other commodities. Its use as a safe fumigant of stored products has become even more important with recent restrictions on the production of the only alternative, methyl bromide. Widespread resistance to phosphine has emerged in several species of stored-product insects in many countries, which in some instances may have caused control failures.

Chemically, phosphine is a strong reducing agent and biological redox systems, especially the components of the mitochondrial electron transport chain, are probably the site of its action in insects. The oxidation of phosphine could produce reactive phosphorylating species and interactions of phosphine with biological redox systems have been reported to cause generation of highly reactive oxyradicals. This appears to be the basis of phosphine toxicity to insects, which differs from that of respiratory inhibitors such as hydrogen cyanide.

Phosphine-resistant strains of several species of stored-product insects have been reported to absorb very small amounts of the compound compared to their susceptible counterparts. This reduced uptake in resistant insects appears to result from respiratory exclusion of phosphine. The overall mechanism of resistance also involves a detoxification process. Despite the likely involvement of oxyradicals in the insecticidal action of phosphine, the level of anti-oxidant enzymes in resistant insects is apparently not higher than that in their susceptible counterparts. The reduced uptake of the compound might be due either to the presence of a phosphine insensitive target site or to a membrane-based efflux system that excludes phosphine gas in resistant insects. Studies have indicated the oxygen uptake in mitochondrial preparations from susceptible and resistant insects to be equally sensitive to inhibition by phosphine *in vitro*. The nature of the phosphine-exclusion system in resistant insects has not been fully elucidated.

The possibilities of controlling resistant insects with phosphine and prospects for developing new alternative fumigants are also discussed.

Key words: phosphine, mode-of-action, resistance, stored-product, insects

1 INTRODUCTION

Phosphine gas (hydrogen phosphide, PH_3) was first used for disinfesting stored products in Germany during the 1930s and the development of metal phosphide formulations, which release the gas *in situ* on exposure to moist air, led to world-wide application of phosphine as a fumigant.[1,2] Phosphine is rapidly diffused in air[3] because it has a similar density to that of air (relative densities 1·13 : 1).[4] Consequently, fumigation with phosphine generally does not require any circulation system for distribution within the fumigated space. Phosphine

fumigation does not produce toxic residues[5–7] and has no adverse effect on the viability of seeds.[8–12] These properties have made the compound the fumigant of choice for protection of stored products across the world and it has virtually replaced several previously used fumigants.[13]

The success of any fumigation operation depends largely on the standard of gas-tightness of the space to be fumigated. The fumigant gas must be retained for a minimum period to be effective against insects; at temperatures >20°C, a three- to five-day exposure of commodities to an effective concentration of phosphine is generally sufficient to control most of the insect pests and their developmental stages, but complete control of some insects, such as *Sitophilus* species, needs exposure for 12 days or longer.[14] The standard of gas-tightness required for effective fumigation is, however, rarely met under practical field conditions found in many countries. An early fall in the concentration of phosphine generally results in the failure of fumigation because surviving insects rapidly rebuild their population in the absence of any residual insect toxicant. Frequent repeated exposures to sublethal levels of the toxicant could also lead to selection of resistant insects.

During 1972–73, a global survey of the susceptibility of stored-product insects to pesticides was undertaken by the Food and Agriculture Organisation of the United Nations.[15] This revealed that almost 10% of the insect populations sampled in different countries included phosphine-resistant individuals. A later survey in Bangladesh, where phosphine fumigations were reported to be failing, showed very high levels of resistance to the compound in many species of stored-product insects.[16,17] It was also shown that the grain stores where repeated fumigations with phosphine had been carried out in the past were not sufficiently gas-tight[16] and under-dosing, resulting from rapid loss of the fumigant gas, was considered to be the cause of build-up of resistant insect populations.[13,17] Other factors contributing to the development and spread of resistance included the use of inadequate amounts of phosphine and movement of resistant insects as a result of international trade in commodities.[18,19]

Further reports have indicated that phosphine resistance occurs in several stored-product insect species and in many countries.[20–31] This is an alarming prospect in view of the exceptional suitability of phosphine as a fumigant for food commodities and also because the future of the only available alternative, methyl bromide, is now in doubt after recent restrictions on production due to alleged deleterious effects on the stratospheric ozone layer.[32] The continued availability of phosphine as a fumigant is, therefore, becoming even more important, especially for use in developing countries where grain is mainly stored in bags and the technology for the application of non-gaseous grain protectants is not available. Due to the importance of phosphine in the post-harvest management of commodities, many improvements in fumigation practices have been suggested to increase efficacy of control of insects (see Section 4). However, none of the methods can be used to control phosphine-resistant insects satisfactorily in practical field situations since resistance, at least in some instances, has reached levels that can lead to control failures even when the best fumigation practices are applied.

This article reviews the mechanisms involved in the toxic action of phosphine and resistance to this fumigant gas in stored-product insects. The prospects for controlling resistant insects and the development of new fumigants are also discussed.

2 MODE OF TOXIC ACTION OF PHOSPHINE

2.1 Physical and chemical properties of phosphine

At room temperature, phosphine is a colourless, flammable gas which, in pure form, is odourless. The garlic-like odour associated with phosphine generated from technical products, perceivable at $1{\cdot}4 \times 10^{-4}$ mg litre^{-1} of phosphine in air,[33] and the formation of explosive mixtures when phosphine concentrations exceed 25 mg litre^{-1} in air,[34,35] have been attributed to the formation of traces of other phosphorus hydrides, particularly the spontaneously explosive diphosphine (P_2H_4).[36,37]

Phosphorus and other elements of group Vb of the periodic table (nitrogen, arsenic, antimony) form trihydride gases of the general formula MH_3. However, compared to ammonia (NH_3) in which the N–H bond has a significant *s* character, the proximity of the H–P–H bond angle to 90° in PH_3 (93° 50′) indicates involvement of only *p* orbitals in bond formation. The lone pair of electrons on phosphorus resides in a relatively diffuse 3*s* orbital and is therefore less available for bonding in PH_3 than is the nitrogen lone pair in NH_3, and for this reason the basicity of PH_3 is much lower than ammonia (pKa values 14 and 9.21 respectively). In alkyl-phosphines, as the *s* bond character increases with the number of alkyl groups, the *p* character of the lone pair also increases making it less diffuse and more available for bonding. Trimethyl phosphine (C–P–C bond angle 99°) is therefore a strong base with a pKa value of 8·65.[38]

The presence of a lone pair of electrons on phosphorus gives the PH_3 molecule a nucleophilic character. The greater size and lower electronegativity of phosphorus compared to nitrogen leads to a high polarisability (3·63 and 1·1 for PH_3 and NH_3 respectively)[38] and thus phosphines have a higher nucleophilic reactivity than their analogous amines. Furthermore, the availability of *d* orbitals on phosphorus enables it to expand the valency to ten electrons. High affinity of phosphines and other P(III) compounds for oxygen is

also due to the expansion of the valence shell and, in this case, by back donation of a lone pair on oxygen to a vacant *d* orbital on phosphorus ($2p\pi$–$3d\pi$). Such reactions are driven by the formation of very strong phosphoryl bonds (bond energy of P=O bond is in the range of 523–631 kJ/mol^{-1}). However, unlike tertiary alkyl-phosphines, PH_3 gas is not autoxidised and PH_3 : air mixtures are relatively stable at standard temperature and pressure (STP).[37] Similarly, whilst alkyl-phosphines undergo autoxidation and oxygen abstraction reactions and their oxides are well known, there is no direct evidence of the existence of a phosphine-oxide of the formula OPH_3.[37] *Ab initio* calculations for an OPH_3 molecule have been reported[39] and Berners-Price and Sadler[40] speculated that even if OPH_3 existed, it would be thermodynamically unstable compared to the tertiary phosphine oxides. Some recent studies, however, suggest that this unstable oxide is probably formed during oxidation of phosphine to various lower-valent oxyacids of phosphorus.[41] Phosphine also acts as a π-acceptor ligand and can bind to transition metal ions. However, phosphine coordinates much less strongly than ammonia because of a smaller permanent dipole and a larger central atom.[42]

Phosphine, unlike ammonia, is not very soluble in water (only 0·26 volumes of phosphine dissolve in 1 volume of water at 25°C)[43] and a saturated solution of phosphine in water at STP is, therefore, 11·6 mM. Aqueous solutions of phosphine are neutral as the PH_3 molecule has no partial charge due to equal electronegativities of hydrogen and phosphorus (2·1 on Pauling's scale).[44] Phosphine, like its group Vb analogues arsine (AsH_3) and stibine (SbH_3), is a strong reducing agent (E_0 for conversion to hypophosphorous acid = 1·18 V)[45] and readily undergoes oxidation in aqueous solutions.[36] Major products resulting from the oxidation of phosphine in water are hypophosphorous and phosphoric acids.[36] The oxy-acids resulting from the oxidation of phosphine cause corrosion of several metals, particularly copper, under conditions of high relative humidity.[46] Phosphine is thermally stable at ambient temperatures and only decomposes when heated above 550°C.[37]

2.2 Possible interactions of phosphine with biological systems

Despite its poor solubility, the amounts of dissolved phosphine in aqueous media are toxicologically significant. It is also important to note that, since the aqueous solubility of phosphine is 6·5 times higher than that of oxygen at STP, it may displace dissolved oxygen in biological fluids.

Phosphine is reported to reduce the S–S bond in cystine,[47] which is comparable to the capability of some organic phosphines to break disulphide bonds in proteins.[48] Biochemically, this could have detrimental consequences, since resulting conformational changes might lead to inactivation of vital proteins. However, whilst phosphine-induced spectral shifts in ferricytochrome-c and ferricytochrome-c oxidase indicated reduction of haem iron to the ferro-form, lack of any change in the circular dichroic spectra suggested that no conformational change in the polypeptide chain occurred in either case.[49]

Comparison of the oxidation potential of phosphorus (P) to other elements of group Vb (Fig. 1) indicates that, with the exception of NH_3 which is basic, all other trihydride gases are strong reducing agents.[24,40,50] Thus, unlike some strong metal-ion ligands, such as cyanide, which bind irreversibly to the haem Fe(III) in the respiratory enzyme cytochrome-c-oxidase complex and to many other metallo-enzymes,[51] the biological effects of phosphine are likely to be dominated by the strong tendency for oxidation to stable P(V) forms. Indeed, the oxidation of phosphine is thermodynamically favourable under physiological conditions at each step of the valency change, and even weak oxidising systems should be able to oxidise it to phosphate.[50] Conversely, the reduction of phosphate to phosphine would require strong reducing systems. This is the reason why phosphorus is mainly present in the pentavalent state in aerobic organisms. Some anaerobic micro-organisms which possess ferridoxins have been reported to reduce phosphate to phosphine, hypophosphite and phosphite during the decomposition of organic matter under waterlogged conditions.[40] Recently, microbial breakdown of phosphate-rich food in mammalian gut has also been shown to produce phosphine gas.[52]

Many reducing agents are known to alter cellular redox balance and disrupt normal oxygen metabolism and this could lead to generation of highly reactive oxy-radicals.[53] Recent evidence suggests that phosphine treatment also initiates this process, which might be the cause of its toxicity to insects (see Section 2.4.3).

2.3 Deviation of phosphine from general principles of fumigant activity

Since fumigants are gases, or highly volatile liquids that exist in gaseous form at ambient temperatures, entry into an exposed animal is believed to be mainly through the respiratory system. The uptake of a fumigant is, therefore, generally proportional to the rate of respiration of the exposed animal and factors which increase respiratory activity should also increase the uptake and toxicity of a fumigant. Thus, increasing the level of carbon dioxide in air to 14% is reported to enhance the toxicity of phosphine to insects through respiratory stimulation.[54] However, the uptake of phosphine in insects essentially requires oxygen (see Section 2.4) and the opening or closing of spiracles has been reported to

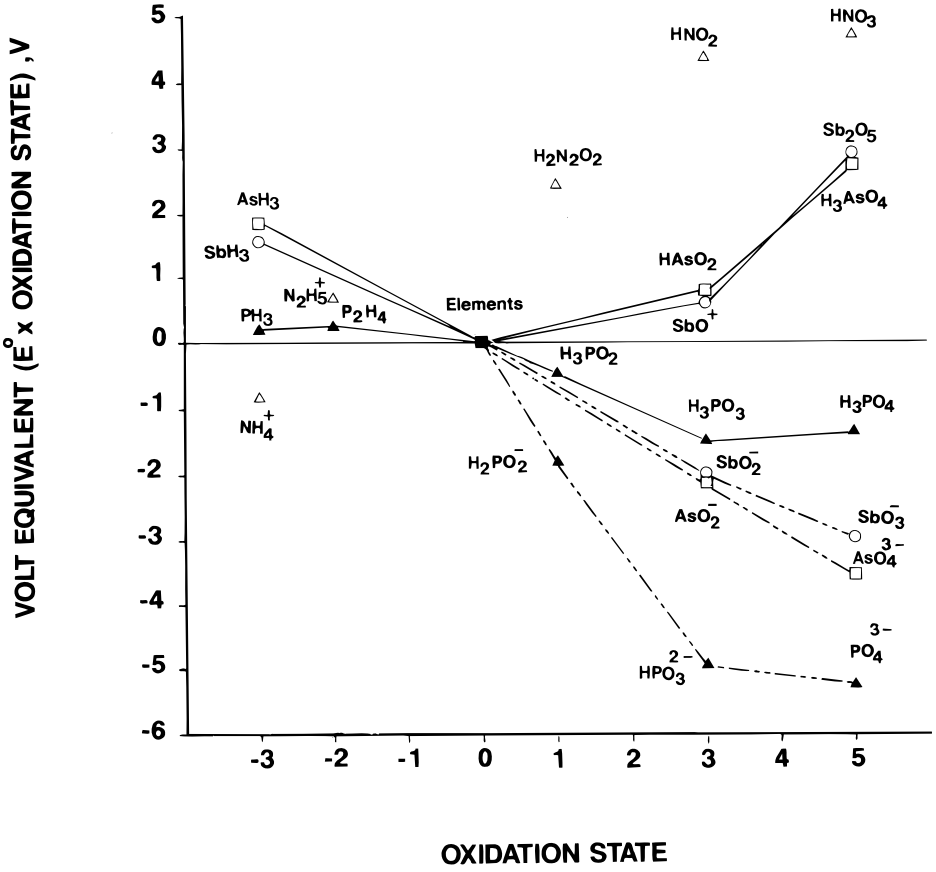

Fig. 1. The oxidation state diagram of group Vb elements, (▲) phosphorus (P), (□) arsenic (As) and (○) antimony (Sb) in acidic (solid lines) and alkaline medium (broken lines) along with selected values of (△) nitrogen (N) in acidic medium. Data from Weast,[4] Latimar[45] and Chaudhry & Price.[50]

have little effect on uptake or resulting mortality in cockroaches.[55] Like other fumigants, the toxicity of phosphine to insects increases with a rise in temperature within the normal range of ambient temperatures,[2,56] probably due to an overall increase in metabolic rate and therefore an increase in oxygen consumption that stimulates the uptake of phosphine.

The pre-adult stages of insects are generally more tolerant to fumigants, due to lower respiratory rates compared to adult insects. Eggs and pupae of several species of stored-product beetles were found to be the stages most tolerant to phosphine.[57,58] Bell[59] found that eggs of four species of stored-product moths, *Ephestia elutella* Huebn., *E. kuehniella* Zell., *E. cautella* Walk. and *Plodia interpunctella* Huebn. were the most tolerant developmental stages of these species. Similarly, eggs of the khapra beetle *Trogoderma granarium* Everts were the most tolerant stage to phosphine at 25°C and 70% R.H.[60] At 20°C and 60% R.H., the diapausing larvae of *T. granarium* were, however, more tolerant than eggs but showed a decrease in tolerance with an increase in temperature. Tolerance to phosphine in insect eggs decreases with the age of the eggs because of an elevation in metabolic rate and respiration as the egg continues to develop with time.[61]

The dosage of a typical fumigant is expressed in terms of the product of its concentration (C) and time of exposure (t). A required dosage rate could therefore be achieved by varying either C or t which, according to Haber's rule within certain extreme limits of the variables, should produce constant toxic effects in a given species of the animal exposed, i.e. Ct is constant.[62] Unlike other fumigants, however, phosphine toxicity deviates from Haber's rule and the relationship between dosage rates and resulting toxicity in insects is not linear. Generally, longer exposure of insects to phosphine, even at low concentrations, produces greater toxicity than shorter exposure to a high concentration.[58] Some deviations from Haber's rule have been explained by modifying the relationship of $Ct = k$, for example:[63]

$$(C - C_0)^n t = k \quad \text{or } C^n(t - t_0) = k$$
$$\text{or } (C - C_0)^n(t - t_0) = k$$

where C_0 and t_0 are the minimum threshold limits of concentration (C) and time of exposure (t) required to achieve a specified level of response, such LD_{99}, in a given species of insect.

It is also interesting to note that the value of n (the toxicity index or the slope of regressions of log t on log

C),[64] not only explains the deviation from Haber's rule but also reflects the mode of toxic action of a fumigant. For example, Bell[65] pointed out that if

- $n < 1$: t_0 is large, indicating an uptake barrier or some other kind of protective mechanism. Recovery from sublethal treatments will be slow in this case.
- $n > 1$: C_0 is large, indicating a detoxification or metabolism of the toxicant. Recovery from sublethal treatments will be rapid.
- $n = 1$: Both C_0 and t_0 are small, suggesting simple cumulative toxicity.

A value of $n = 0{\cdot}9$ has been reported for the diapausing larvae of *E. elutella* in a dose range of 0·04–0·35 mg litre^{-1} of phosphine.[66] At concentrations higher than 0·35 mg litre^{-1}, the value of n was 0·4 and therefore the time of exposure was the critical determinant of toxicity. The insecticidal action of phosphine is therefore marked by the importance of exposure time, which presumably reflects the time required for the toxicant to reach the site of action and to saturate available target sites in insects. In general, increasing the concentration of phosphine may not necessarily produce greater effects, since several species of stored-product insect are capable of undergoing a state of protective stupefaction or 'narcosis' at higher concentrations of the compound.[55,67,68] It has been suggested that phosphine-induced narcosis provides some protection to insects from the toxic effects because of a lowering of respiration and metabolic activity, especially during short exposures.[64,69]

2.4 Physiological and biochemical aspects of phosphine intoxication

Although phosphine intoxication produces a variety of physiological responses in humans, test animals and insects, the predominant feature in all cases is the inhibition of aerobic respiration. In humans and other mammals, the symptoms are complicated by the reaction of phosphine with the components of blood, and some of the effects may be secondary to hypoxemia. Mild exposure of humans and test animals to phosphine produces symptoms including sleepiness, headache, weakness, respiratory distress and disturbed circulatory regulation.[70,71] Severe phosphine poisoning is marked by restlessness, epigastric pain, vomiting, difficulty in breathing (dyspnoea) and cessation of breathing (apnoea), circulatory breakdown, anoxaemic convulsions, pulmonary edema, cardiac arrest and death. Postmortem examination of several test animals killed by exposure to phosphine revealed congestion in all organs and severe histological damage in kidneys, liver and brain.[70] This was interpreted as 'complex osmotic and permeability disturbances',[72] but the changes resembled those produced by anoxia and may be secondary to hypoxemia.[70]

Trimborn and Klimmer[73] reported that the reaction of phosphine with haemoglobin in the presence of oxygen formed a brown pigment in the blood of phosphine-treated animals. Methaemoglobin was not formed in similar experiments, and the brown coloration of blood was described as the 'formation of a series of verdichromogen-like substances.[70] The significance of this reaction to the toxicity of phosphine was, however, discounted despite a considerable decrease (up to 50%) in haemoglobin and erythrocyte count in the blood of test animals after in-vivo poisoning by the compound.[70] Unlike arsine, low concentrations of phosphine do not cause haemolysis in test animals[74] but may still cause hyperaemia, especially in lungs, kidneys and brain.[75] A spectral study of the effects of phosphine on haemoglobin and myoglobin indicated slow deoxygenation of both of the oxygen-carrier proteins by phosphine, *in vitro*,[76] indicating a high affinity of the compound for oxygen bound to proteins. There is no diagnostic test available for phosphine poisoning; as a consequence, the effects of the rodenticide zinc phosphide, which releases phosphine in mammalian gut, are not distinguishable from similar effects caused by nervous system toxicants in test animals.[77] Following mild exposure to aluminium phosphide, another phosphine-generating compound, 20 out of 30 patients had higher plasma cortisol levels due to effects on the adrenal cortex.[78]

Insects do not have oxygen-binding proteins in the haemolymph and get a direct supply of oxygen to their tissues through a well-developed system of respiratory tubes or tracheae which further branch to form a network of fine capillary-like tracheoles.[79,80] Bond *et al.*[55] described the symptomology of phosphine intoxication in insects. The first signs of intoxication in insects were trembling of legs and palps and closure of thoracic spiracles followed by a sharp decline in oxygen consumption. Later symptoms, depending on the severity of the exposure, included loss of coordination, knockdown, paralysis, respiratory inhibition, cessation of heart beat and whole-body convulsions which eventually diminished as the insects became moribund. Microscopic observations of cockroaches exposed to phosphine showed that, although insects kept their spiracles closed in an atmosphere of air or oxygen, this apparently did not affect the uptake of phosphine or resulting mortality, whereas exposure to phosphine in a nitrogen atmosphere, where spiracles were kept open, resulted in no uptake or mortality.[55] This clearly indicated that oxygen is essential for the uptake of phosphine in insects. The later observation made by Banks[81] that absorbed [^{32}P]-radiolabel in $^{32}PH_3$-treated insects was not desorbed on airing, further suggested oxidative conversion of phosphine in insects. It is now well established that the uptake of phosphine and its action in insects requires oxygen.[82–85] It has also been demonstrated that phosphine is not absorbed by insects in the

absence of oxygen and there is then no insecticidal action.[55,85] A higher mortality after phosphine treatment of the granary weevil *Sitophilus granarius* L. and the rust-red flour beetle *Tribolium castaneum* Hbst. has been reported during post-treatment exposure to oxygen, compared to similarly treated insects kept in air.[82,86] This demonstrates a crucial role of oxygen in the insecticidal action of phosphine, possibly involving the generation of highly reactive oxyradicals (see Section 2.4.3).

Multiple treatments with phosphine do not produce cumulative effects in mammals, but repeated exposures to sub-lethal doses produce accumulation of toxic effects in insects.[86,87] It has been suggested that decreases in the fecundity of *T. castaneum* after phosphine treatment were due to greater toxic effects on female than on male insects.[64,88] Sub-lethal doses of phosphine have also been reported to cause sterility in adult *E. cautella* emerging from treated pupae.[89] Al-Hakkak[90] reported phosphine-induced sex-linked recessive lethal mutations in the fruit-fly *Drosophila melanogaster* Meig.

Phosphine fumigation does not have adverse effects on the germination of seeds of low moisture content (10–11%) but has been shown to cause chromosomal aberration and mitotic inhibition in various seeds under high moisture conditions.[91,92]

2.4.1 Effects on respiratory enzymes

The action of phosphine in insects has long been believed to resemble that of a respiratory poison, since the inhibition of respiration is an essential feature of phosphine intoxication in insects and mammals. Biochemically, molecular oxygen is used by aerobic organisms as an acceptor of the metabolically generated electrons in the mitochondria. Several flavoproteins and haemproteins (cytochromes) form an ion-gradient with a series of redox potentials and, by reversible oxidation and reduction, transport electrons to the terminal enzyme, cytochrome-c oxidase, which catalyses their transfer to oxygen. This process is vital for all aerobic organisms, since it is coupled to oxidative phosphorylation, which is the energy-generating process that converts ADP to ATP.

Nakakita *et al.*[93] reported inhibition of oxygen consumption in mitochondrial preparations from rat liver after treatment with phosphine *in vitro*. The use of succinate or pyruvate and malate as substrates showed a much greater inhibition of mitochondrial respiration in the substrate active state (state-III) than in the resting state (state-IV). Also the inhibitory effect of phosphine was much greater on sonicated particles than on the intact organelles. Nakakita[94] demonstrated the inhibitory action of phosphine *in vitro* on state-III respiration of mitochondria from rat liver and *S. granarius*, both in the ion-pumping and uncoupled states. On the basis of spectral difference between the untreated and phosphine-treated mitochondrial preparations, a direct inhibitory action of phosphine on cytochrome-c oxidase was proposed.[94] Chefurka *et al.*[95] demonstrated that phosphine treatment inhibited oxygen consumption in mitochondria isolated from granary weevil, house-fly flight muscle and mouse liver. Using artificial substrates, which divert the flow of electrons at different sites of the respiratory chain, the authors,[95] in common with an earlier report,[94] demonstrated a much greater inhibitory action of phosphine on state-III respiration than on state-IV and a more pronounced effect on sonicated particles than on the intact mitochondria. Phosphine treatment did not have any in-vitro effects on ATPase activity and exchange of either ATP-Pi or ATP-ADP. These authors[94,95] also suggested that the terminal enzyme complex, cytochrome-c oxidase, was the probable target site of phosphine in insects. The toxic action of phosphine was therefore considered to be similar to that of cyanide, a known inhibitor of cytochrome-c oxidase. This hypothesis was further supported by changes in the absorption spectra of both ferricytochrome-c and ferricytochrome-c oxidase following treatment with phosphine.[49] The spectral shifts in either case indicated that the reduction of Fe(III) to Fe(II) resulted in a conformational change in the haem moiety as evidenced by the circular dichroic spectra. The data did not, however, indicate conformational changes in the polypeptide chain in either case. A greater reducing action of phosphine on cytochrome-c oxidase than on cytochrome-c was indicated and the authors[49] supported the earlier hypotheses[94,95] that cytochrome-c oxidase was the likely target site of phosphine in insects. Since ferrocytochrome-c oxidase is directly oxidised by oxygen and is only then reducible with phosphine, this agreed well with earlier findings that oxygen was essentially required for the toxic action of phosphine in insects.

Contrary to the strong effects *in vitro* on mitochondrial respiration, phosphine treatments did not appear to cause any appreciable inhibition *in vivo* of cytochrome-c oxidase activity in the lesser grain borer *Rhyzopertha dominica* F, although the insects were severely poisoned by phosphine.[96,97] Similarly, treatment of *S. granarius* with phosphine *in vivo* caused only 50% reduction in mitochondrial respiration.[98] The biochemical effects produced by phosphine in *R. dominica* were also different from those resulting from other treatments that are known to block the respiratory electron transport chain, i.e anoxia and hydrogen cyanide.[99] Whilst the level of anaerobic metabolites pyruvate and lactate increased in *R. dominica* after exposure to anoxia, and the level of pyruvate increased after treatment with hydrogen cyanide, no accumulation of either metabolite resulted after phosphine treatment.[99] The only similarity between the biochemical effects of hydrogen cyanide and phosphine treatments in *R. dominica* was the lowering of ATP levels observed

following exposure to sublethal doses of the two gases. Also, unlike phosphine, the exposure of *E. cautella* eggs to anoxia caused delay in development and surviving embryos showed ultrastructural lesions such as changes in the mitochondrial organisation.[100] Pre-treatment of *T. castaneum* with hydrogen cyanide, a known inhibitor of the mitochondrial respiratory chain, did not potentiate the effects of phosphine,[86] supporting the hypothesis that the latter may not be a direct inhibitor of the mitochondrial respiratory chain in insects.

The cytochrome P_{450}-mediated microsomal mixed function oxidases (mfo) have been implicated in the oxidative detoxification of insecticides in insects and mammals.[101] This important family of haemproteins has also been implicated in the oxidative activation of various toxicants and drugs.[102] The possible role of cytochromes P_{450} in the toxicity or detoxification of phosphine has also been studied. Treatment of *T. castaneum*, *S. granarius* and the house fly *Musca domestica* L. with the insecticide synergists piperonyl butoxide and SKF525A (2-(diethylamino)ethyl 2,2-diphenylvalerate), that are known to inhibit microsomal oxidase enzymes, produced only minor increases in the toxicity of phosphine and any significant role of microsomal oxidases in the detoxification of the latter was discounted.[103] This is in contrast to diethylphenyl phosphine (DEPP) which reacted with 83% of the oxidised cytochrome P_{450} in microsomal preparation from phenobarbital pre-treated rats.[104] The difference spectrum showed formation of a complex and its reduction with dithionite produced a Soret band at 459 nm,[104] indicating the involvement of mixed function oxidases in the metabolism of organic phosphines. As insect haemolymph does not have oxygen-binding proteins, carbon monoxide (CO) gas acts as a specific inhibitor of insect microsomal cytochromes P_{450}. However, whilst treatment of insects with 40% CO atmosphere has been reported to greatly enhance the toxicity of several insecticides,[105,106] this did not produce appreciable changes in the susceptibility of *R. dominica* strains to phosphine.[24] It therefore appears to be unlikely that the mfo system plays any significant role in the toxic action of phosphine in insects.

2.4.2 Effects on antioxidant and metabolic enzymes

Phosphine treatment of insects, mammals and grains has been shown to inhibit a variety of enzymes, although, in most cases, the significance of the inhibition to the activity of phosphine has not been established. Reduction in the activity of catalase, a defensive antioxidant enzyme which protects aerobic cells from the toxicity of hydrogen peroxide (H_2O_2), has been shown after phosphine treatment of *S. granarius*,[82,107] *R. dominica*[97,108] and *T. castaneum*.[86] Bond[82] reported that several fumigants inhibited catalase activity in insects after exposure *in vivo* but that the effect was much more pronounced by treatment with phosphine or hydrogen cyanide. However, he discounted the significance of catalase inhibition in enhancement of the toxic action of fumigants by oxygen. Bolter and Chefurka[107] demonstrated that peroxidase, another antioxidant enzyme and catalase were inhibited in *S. granarius* treated with phosphine. It was postulated that inhibition of catalase activity was the possible cause of phosphine toxicity to insects since this would lead to accumulation of cytotoxic hydrogen peroxide in insect tissues.[86,107,108] However, in contrast to the ability of phosphine to inhibit insect catalase *in vivo*, only artificially high doses produce any significant inhibition of this enzyme *in vitro*.[86] Price and Dance[97] found that phosphine did not appreciably decrease catalase activity *in vitro* in homogenates prepared from four species of stored-product beetles, and suggested that inhibition *in vivo* was an indirect secondary effect of phosphine activity rather than a cause of it. This is supported by the findings that, although dietary 3-amino-1,2,4-triazole (a semi-specific inhibitor of catalase biosynthesis) lowered the level of catalase in *R. dominica*, there was no significant effect on the susceptibility of these insects to phosphine.[99] The significance of catalase inhibition to the toxicity of phosphine in insects is therefore questionable. In fact, this is most probably the result of oxyradical generation, a process that has been reported to inhibit both catalase and peroxidase activity.[53,109]

Pazynich *et al.*[110] reported that blood cholinesterase, catalase and peroxidase were inhibited in rats exposed to sublethal doses of phosphine over a period of 1·5 months. Changes in serum glutamic-pyruvic and glutamic-oxaloacetic transaminases, leucine aminopeptidase, aldolase, alkaline phosphatase and albumin have been reported in rabbits poisoned with zinc phosphide.[74] Inhibition of catalase, glutamic acid decarboxylase and alcohol dehydrogenase have been reported in wheat fumigated with phosphine.[111] Also, whilst many other fumigants caused decreases in the level of cytosolic glutathion in insects, no such depletion resulted from treatment with phosphine or carbon tetrachloride.[112]

Al-Hakkak *et al.*[113] reported that phosphine treatment of *E. cautella* inhibited acetylcholinesterase activity both *in vivo* and when homogenates prepared from pupae of these insects were treated *in vitro*. However, the doses of phosphine used in the *in vitro* study were extremely high (0.063–0.333 mg ml^{-1}) and the effects shown might well have been artefacts caused by the large amounts of solubilised phosphine or its oxidation products.

2.4.3 Oxidative metabolism of phosphine and generation of oxyradicals

The main feature of phosphine metabolism in insects and other animals is the oxidative conversion of reactive P(III) to stable P(V) oxyacids of phosphorus,

mainly hypophosphite, phosphite and orthophosphate.[47,114] Although quantitatively different, this process has been shown to be qualitatively similar in susceptible and phosphine resistant insects.[114,115] Nakakita and Kuroda[116] attributed the uptake of phosphine by *T. castaneum* to the presence of a cytosolic factor which caused absorption of both oxygen and phosphine *in vitro*. This factor was reported to be stable over a pH range of 5–7 and up to 50°C.[117] Later studies indicated, however, that it may have been an artefact due to the presence of defensive quinones in *Tribolium* species, as there was no evidence for the presence of such a factor in *R. dominica*, despite a comparable uptake of phosphine by this species.[115]

Interactions of phosphine with components of the mitochondrial electron transport chain are well established, and possibly result in oxidative metabolism of the compound in insects. Hypophosphite has been reported to be the main metabolite excreted in the urine of test animals treated with phosphine[118] and Lam *et al.*[41] reported the presence of hypophosphite and phosphite in the urine of rats after oral administration of zinc phosphide. The major oxidation product of phosphine, hypophosphite, is non-toxic to various test animals.[36] In man, hypophosphite and dilute hypophosphorous acid have been used as a dietary supplement of phosphorus in debilitated conditions and in convalescence, although the hypophosphite ion is reported to pass through the body unchanged.[119] In insects, a 21–34% increase in the median and maximum life spans of the drosophilid fly *Zaprionus paravittiger* (Godble & Vaidya) has been reported after feeding on diets containing a range of concentrations of sodium hypophosphite.[120] The non-toxic nature of phosphine metabolites does not explain why phosphine is a potent insecticide and it is likely, therefore, that interactions of phosphine with biological redox systems lead to the production of reactive species in insects. Chemical studies have indicated that the oxidation of phosphine potentially generates reactive electrophilic species,[41] and, although the significance of this process to the toxicity of the compound *in vivo* is not known, these intermediates may cause phosphorylation of vital bionucleophiles. As the strong tendency for oxidation to P(V) dominates interactions of the PH_3 molecule, Berners-Price and Sadler[40] speculated that it could lead to the generation of oxyradicals under aprotic conditions, such as in the vicinity of membranes. It is well established that disruption of normal 4-electron reduction of dioxygen by the mitochondrial respiratory chain results in partial reduction to highly reactive oxyradicals. Such a process could lead to severe detrimental consequences in living systems, such as alteration of cellular redox balance, peroxidation of lipids and other tissue components and inactivation of vital proteins.[121] It has been reported that treatment *in vitro* of insect mitochondria with phosphine resulted in increased levels of hydrogen peroxide.[122] This evidence, coupled with elevation in superoxide dismutase (SOD) activity in *S. granarius* after sub-lethal treatment with phosphine *in vivo*, led to the hypothesis that generation of oxyradicals is involved in the activity of the latter against insects.[107]

Phosphine treatment of *R. dominica* has also been reported to cause a lowering of the activities of catalase and peroxidase and an increase both in SOD activity and level of lipid-peroxides.[24,123] These findings strongly indicate that phosphine causes the generation of highly reactive oxyradicals in insects, which also explains the reason why oxygen is essential for the toxic action of phosphine in insects.[82–86]

3 MECHANISMS OF INSECT RESISTANCE TO PHOSPHINE

Early reports of phosphine tolerance in insects were based on selection of resistant insect strains in the laboratory. Monro *et al.*[124] reported a three-fold increase in tolerance to phosphine in a strain of *S. granarius* after 28 generations of selection, and Winks[125] reported a 6·5-fold increase in the tolerance of a twice-selected strain of *T. castaneum*. Kem[126] and Saxena and Bhatia[127] reported 12- and 5·9-fold increases in tolerance of adult *T. castaneum* after 10 and 16 generations of selection respectively. Selection for high levels of methyl bromide resistance in laboratory strains had been difficult compared to selection for phosphine resistance[128] and Winks[129] speculated that either genes for phosphine resistance were already present at low frequencies or that phosphine treatment caused high rates of mutation. Winks[129] also reported that there was no appreciable change in the level of phosphine resistance in a laboratory-selected strain of *T. castaneum* maintained for 17 years without any further selection.

As described above, the occurrence of phosphine-resistant insects in the field was first detected during a world-wide FAO survey in 1972–73.[15] The greatest incidence and highest levels of resistance ($\times 10$) were reported in *R. dominica* and two species of *Tribolium*.[15,130] Laboratory selection of some of the field strains further increased levels of resistance to phosphine.[129,131] However, much higher levels of resistance were subsequently recorded in field strains of several species of stored-product insects from Bangladesh.[16,17,131] For example, in comparison to a complete kill of susceptible *R. dominica* after 20 h exposure to 0·03 mg phosphine litre^{-1} (a discriminating dose for this species),[132] all individuals of a strain originally collected from Bangladesh could survive 1·45 mg litre^{-1} for 20 h, and exposure to this dose for 72 h was required to kill all insects of this strain.[133] At least 11 species of stored-product insects are now known to have developed resistance to phosphine, 10 of them beetles and one moth species (Mills, K. A. pers.

commun.). A strain of the psocid *Liposcelis* species from India survived seven days exposure to 1·5 mg phosphine litre^{-1}, although further work is needed to establish the true nature of resistance in this species (Mills, K. A., pers. commun.).

Pre-adult stages of several species of phosphine-resistant insects are also reported to have a higher tolerance to phosphine than the corresponding stages of susceptible conspecifics.[134] The eggs of resistant strains of *R. dominica*,[135] and the eggs, larvae and pupae of *T. granarium*[136] were also reported to be phosphine-resistant, but assessment of mortality in the latter study may have been inadequate.[60,129]

3.1 Differences between susceptible and resistant strains of insect

Phosphine-induced narcosis has been proposed as a protective mechanism in insects, and a lowering of the threshold to narcosis was postulated as a possible mechanism of phosphine resistance.[125,137] Winks[69,129] and Winks and Waterford[88] studied the response of *T. castaneum* strains to a wide range of phosphine concentrations and exposure periods. The toxicity index (*n*, see Section 2.3) at the LD_{50} level varied from 0·65 at a range of 0·025–5·0 mg phosphine litre^{-1} to 1·3 at the range of 0·0095–0·025 mg litre^{-1}, compared to 0·89 for the susceptible strain. From this, Winks and Waterford[88] concluded that the toxicity indices for susceptible and resistant strains were different, and that it would be misleading to derive a single resistance factor from either a fixed concentration or exposure time. Their studies also discounted narcosis as a resistance mechanism in *T. castaneum*, since a resistant strain had a higher, instead of the expected lower, narcosis threshold compared to a susceptible strain.[88] In fact, a rapid test for phosphine resistance has been developed recently based on quicker knockdown of susceptible insects, at predetermined concentrations of phosphine, compared to their resistant counterparts.[138]

It has also been reported that exposure to phosphine caused greater inhibition of respiration in susceptible strains of *R. dominica*[96] and *T. castaneum*[116] than in their resistant counterparts. Further studies, however, failed to show any appreciable differences in the sensitivity of mitochondrial preparations from susceptible and phosphine-resistant strains of *R. dominica* to inhibition by phosphine.[24,139] Thus, it is unlikely that a phosphine-insensitive mitochondrial respiratory chain is the major mechanism of phosphine resistance, and the differences in respiratory inhibition are probably due to a different level of phosphine uptake (see Section 3.2).

Kashi[84] proposed a correlation between survival of anoxia in five species of stored-product insects and their tolerance to phosphine, although the data presented did not fully support the hypothesis. Similar experiments using *R. dominica* showed that there was no correlation between phosphine resistance status and survival under anoxic conditions.[24]

Bell[140] reported that high concentrations of phosphine (>0·5 mg litre^{-1}) were repellent to a phosphine-susceptible strain of *S. granarius* but not to a resistant strain, and that both susceptible and resistant strains of the saw-toothed grain beetle *Oryzaephilus surinamensis* L. were attracted to phosphine rather than repelled. In addition, a resistant strain of the rust-red grain beetle *Cryptolestes ferrugineus* (Steph) showed no response, whilst a resistant strain of *T. castaneum* was attracted to the increasing concentrations of phosphine. Adult beetles of the species *T. castaneum* that were resistant to phosphine or malathion appeared to be more active than a susceptible strain, whilst no such difference was observed in lindane- or DDT-selected strains.[141] It has also been reported that phosphine-resistant strains of *T. castaneum* were more tolerant to gamma radiation than the susceptible strain.[142] Phosphine-resistant and susceptible adults of the species *T. castaneum* were killed by 200 and 150 Gy respectively. Udeaan and Judge[143] found no significant difference between the life cycle characteristics of susceptible and phosphine-resistant *T. granarium*, except that the larval period was longer in resistant than in susceptible insects.

There is no evidence of cross-resistance between phosphine and other fumigants[124,144] or pesticides,[145,146] although some strains have been reported to exhibit multiple resistance to pesticides and phosphine.[145]

3.2 Differences in the uptake of phosphine between susceptible and resistant strains of insects

The use of radiotracers and other analytical techniques has indicated that the mechanism of phosphine resistance in insects is probably related to reduced uptake of the compound. This has been demonstrated in *R. dominica*,[114,147–149] *T. castaneum*,[24,116,149] *C. ferrugineus*[97] and *O. surinamensis*[97] (Fig. 2). The fact that some of the strains were collected from different countries indicates a common mechanism of phosphine resistance in these beetles. The pre-adult stages of a phosphine-resistant strain of *T. castaneum* also had reduced uptake of the compound compared to that in the corresponding stages of a susceptible strain.[116]

Reduced phosphine uptake does not appear to be due to lowering of respiration, as the consumption of oxygen in resistant insects is unaffected at doses of phosphine that severely inhibit respiration in their susceptible counterparts.[96,116] However, severe inhibition of respiration in susceptible *T. castaneum* by phosphine had no apparent effect on phosphine uptake and Nakakita and Kuroda[116] speculated that phosphine passively diffused through the insect integument, although there

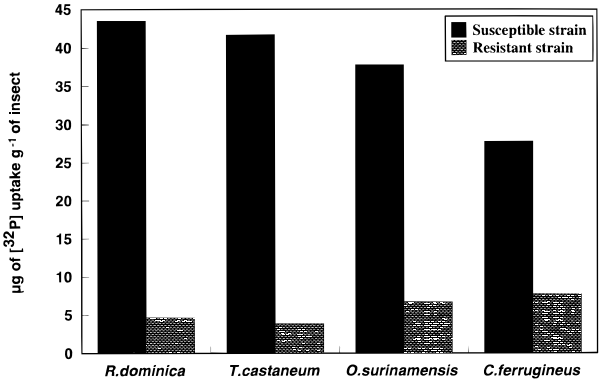

Fig. 2. The uptake of $^{32}PH_3$ in susceptible and phosphine-resistant strains of four species of stored-product beetles. The strains of *Rhyzopertha dominica* and *Tribolium castaneum* were treated at 0.7 mg $^{32}PH_3$ litre^{-1} for 5 h and *Oryzaephilus surinamensis* and *Cryptolestes ferrugineus* at 1·25 mg litre^{-1} for 4 h. (Data from Chaudhry[24] and Price & Dance[97]).

is no evidence to support this suggestion. The amounts of [^{32}P]-radiolabel absorbed by resistant *R. dominica* after treatment with $^{32}PH_3$ were even lower than those taken up passively by dead insects, and Price[147] regarded the phenomenon as an 'active exclusion' of phosphine. Whilst a rise in temperature (5 to 30°C) during a five-hour exposure to $^{32}PH_3$ enhanced the uptake of [^{32}P] in susceptible *R. dominica*, resistant strains showed an apparent increase in the process of [^{32}P] exclusion.[147] A pulse-chase study also showed that the exchange of gaseous [^{32}P] in $^{32}PH_3$-treated resistant strains of *R. dominica* was higher than in a susceptible strain and was the possible cause of reduced levels of [^{32}P] uptake by resistant insects.[123] Despite quantitative differences, the oxidation products of $^{32}PH_3$ in treated insects have been shown to be qualitatively similar in phosphine-resistant and -susceptible strains of *R. dominica*.[114,115] In comparison to a susceptible strain of *R. dominica*, resistant insects were reported to excrete considerable amounts of [^{32}P]-radiolabel during longer exposure to $^{32}PH_3$.[114,148] However, this was possibly due to their greater survival during longer exposures to phosphine than the susceptible insects, permitting the opportunity to excrete metabolised radiolabelled products. Many previous studies did not take account of large differences in the uptake of phosphine, so that reported effects such as lower inhibitory effects of the compound on insect respiration[96,116] and enzyme activity[86,107] in resistant insect strains, compared to susceptible insects, may have been due to a reduced uptake of phosphine.

Further studies have revealed that although reduced uptake of phosphine is a major mechanism, it is not the only process involved in phosphine resistance. Treatment of resistant strains of *R. dominica* with higher doses of $^{32}PH_3$ to induce uptake equal to that recorded in the susceptible strain, failed to produce equi-toxic effects.[148] Negligible mortality in phosphine-resistant strains compared to complete mortality of the susceptible insects clearly indicated the existence of a detoxification process in addition to the reduced uptake of phosphine in resistant insects. It is also interesting to note that similar levels of uptake, achieved over different lengths of exposure to phosphine, produced different effects in resistant insects. Thus, an equivalent uptake achieved over longer exposure to a lower concentration of $^{32}PH_3$ resulted in much higher mortality of resistant insects compared to negligible effects during short exposure to a high concentration of the gas.[148] Also, despite the likely involvement of oxyradical generation in the toxic action of phosphine in insects, the levels of antioxidant enzymes in resistant strains of *R. dominica* were not appreciably higher than those in susceptible conspecifics.[123] Even at doses that induced equal uptake of phosphine in susceptible and resistant strains, the effects of oxyradical generation were evident only in susceptible insects.[123] This further demonstrates that the overall mechanism of phosphine resistance in insects involves exclusion of the gas *via* the respiratory system and a supporting detoxification process capable of converting it to non-toxic oxyacids before reaching the target site. Recently, conventional genetic studies in different species of stored-product insects have also indicated that two or more genes are involved in phosphine resistance (Mills, K. A. pers. commun.).[150,151]

The exact nature of the phosphine-exclusion mechanism in insects is not known. Based on the chemical properties of the compound and its tendency for oxidation, it has been speculated that a system exists in tracheal membranes to exclude the gas before it reaches the cellular target site.[24] Preliminary work on some genes that encode for efflux systems, such as multi-drug resistance (mdr) proteins involved in active exclusion of a variety of xenobiotics, has indicated that such systems are probably not involved in phosphine resistance in insects (Chaudhry, M. Q., unpublished).

4 CONTROLLING RESISTANT INSECTS WITH PHOSPHINE AND PROSPECTS FOR DEVELOPING NEW FUMIGANTS

The problem of phosphine resistance in insects has led many workers to devise improved methods of fumigation to enhance the efficacy of phosphine for their control, and also to search for suitable alternatives to this fumigant. There is, however, an intrinsic handicap to the development of a new fumigant due to a very limited choice of suitable insecticidal compounds that can exist in gaseous form under normal conditions of temperature and pressure. Also, recent concerns about the environmental and health effects of pesticides in general have led to further shortening of the list of possible candidate compounds. Thus, whilst the application of many previously used fumigants, such as hydrogen

cyanide, carbon disulphide and some alkene halides has been abandoned, the non-toxic nature of phosphine residues has led to its continued world-wide use a fumigant.

As discussed above, the mechanism of phosphine resistance in insects appears to involve respiratory exclusion of the gas coupled with detoxification. Resistant insects can apparently survive high concentrations of phosphine over short exposure periods but are killed during extended exposures to lower concentrations of the gas.[58,147,148] Thus, the effects of phosphine appear to accumulate slowly in resistant insects[24,123,148] and it is possible that the resistance mechanism is gradually saturated during longer exposures. The main strategy to control phosphine-resistant insects with phosphine would therefore involve extending the exposure periods rather than increasing the concentration of the fumigant gas. This essentially requires application of phosphine in a gas-tight environment,[152] which is difficult to achieve under practical field situations in some developing countries, despite the design of suitable stores[153] and methods for sealing storage structures for fumigation.[154] Fumigation of commodities under gas-proof sheets is one example of how exposure to phosphine can be extended.[155] Phosphine fumigation of relatively dry grain under polyethylene sheets and keeping the grain sealed for the rest of the storage period to avoid re-infestation, has been recommended to maximise the duration of insect-free storage.[156,157] Other improvements involve compensating for the loss of fumigant gas through leakage by applying phosphine in multiple doses,[158] use of formulations that release the gas at a slower rate[159] or maintaining a low level of phosphine by continuous supply from a cylinder.[160] As described above, presence of about 14% carbon dioxide in air has been reported to stimulate respiratory activity and enhance the uptake and toxic effects of phosphine in insects.[54] A mixture of phosphine in carbon dioxide has therefore been used to enhance the fumigant action of the former against some species of stored-product insects,[161–163] although its usefulness for controlling resistant insects is doubtful. Since fumigants are mainly absorbed by insects through the respiratory system, the most sensitive target for a candidate fumigant would be the components of the respiratory chain in the mitochondria. Recently, the fumigant properties of carbonyl sulphide gas (COS) have been reported.[164] The insecticidal action of COS has been thought to arise from its hydrolysis by carbonic anhydrase to produce hydrogen sulphide (H_2S) gas in insects. However, the high aqueous solubility of COS and breakdown of solubilised gas means that it may not be a suitable alternative to phosphine, since its application might lead to sulphurous residues in fumigated commodities.

Chaudhry and Price[50] reported that arsine (AsH_3) and stibine (SbH_3) caused much greater mortality of phosphine-resistant insects than of susceptible ones. However, these gases could not be used as fumigants due to the probability of toxic residues. Nevertheless, the greater mortality of phosphine-resistant insects caused by arsine and stibine indicated that the resistance mechanism is unable to exclude other molecules that are chemically similar to phosphine, and that their breakdown by the detoxification process resulted in formation of toxic products (arsenite and antimonite). Silane (SiH_4) and methylsilane (CH_3SiH_3) were also considered to be potential new fumigants as their breakdown would produce non-toxic silicon oxides. Unfortunately, both of these gases had relatively little activity against insects compared to the trihydride gases of group Vb elements i.e. PH_3, AsH_3 and SbH_3 (Chaudhry, M. Q., unpublished). Recent studies at Central Science Laboratory, York, UK have led to the discovery of fumigant properties of methylphosphine gas (CH_3PH_2), a close analogue of phosphine.[165] In preliminary trials, methylphosphine caused a much greater mortality of phosphine-resistant insects from four species of stored-product beetles compared to their corresponding susceptible counterparts.[166] It is likely that the presence of the methyl group in methylphosphine prevents exclusion by the phosphine resistance mechanism, and that methylphosphine is activated by the enzymes that detoxify phosphine in resistant insects. Further studies are needed to establish the nature and extent of residues arising from methylphosphine fumigation of commodities, although comparison with phosphine indicates that they would be non-toxic. As methylphosphine is a gas, it could be used as a fumigant alone or in mixtures with phosphine.

The exceptional suitability of phosphine as a fumigant, and its world-wide importance in the post-harvest protection of foodstuffs, necessitates additional efforts to overcome the problem of resistance in insect pests. Although research so far has given important clues as to the nature of phosphine resistance in insects, further studies are needed to broaden our understanding of this novel mechanism of resistance and to develop management strategies. Improvements in the application of phosphine under field situations, especially in less developed countries, are also needed to avoid further selection of phosphine resistance. Considering the scarcity of choice among suitable gases, it is unlikely that many novel fumigants will be available in future. If the use of the newly discovered fumigant methylphosphine, meets current expectations with respect to selective control of phosphine-resistant insects, it will however, offer a new hope to improve the efficacy of fumigations, thus extending the useful life of phosphine as a fumigant.

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